--Microorganisms capable of producing a single cell oil containing DHA are cultivated in a fermentor in a nutrient solution capable of supporting the growth of such organisms. Preferably the single cell oil will contain at least about 20% by weight DHA.

Any microorganisms capable of producing a single-cell edible oil containing DHA can be used in the present invention. For example, photosynthetic diatoms can be used. Preferred microorganisms are marine dinoflagellates, including *Crypthecodinium sp.* Especially preferred is *Crypthecodinium cohnii*, an obligate heterotroph requiring a reduced carbon source for growth. *C. cohnii* is preferred because it contains a fatty acid profile in which DHA is the only PUFA present in sufficient quantities (greater than about 1% of the total amount of PUFAs). Samples of this organism, designated MK8840, have been deposited with the American Type Culture Collection at Rockville, Maryland, and assigned accession number 40750. As used herein, microorganism, or any specific type of microorganism, includes wild strains, mutants or recombinant types. Any microorganism which produces enhanced levels of oil containing DHA is considered to be within the scope of this invention. One of the features of the present invention is its recognition of the edible oil-producing capability of microorganisms such as dinoflagellates and the attendant solution to the problem of maintaining a reliable, economic source of such oils. Accordingly, wild-type and recombinant microorganisms designed to produce single cell oil containing DHA are an aspect of this invention. Such recombinant organisms would include those designed to produce greater quantities of DHA in the single cell oil, greater quantities of total oil, or both, as compared to the quantities produced by the same wild type microorganism, when provided with the same substrates. Also included would be

microorganisms designed to efficiently use more cost-effective substrates while producing the same amount of single cell oil containing DHA as the comparable wild-type microorganism.

In general, those of skill in the art would not consider *C. cohnii* a suitable organism for cultivation in a fermentor. Previous workers have commented on the extremely complex mixture of nutrients required to successfully cultivate *C. cohnii*. Gold et al. *Protozoal*, 13:255-257 (1966); Guillard, et al. in "Dinoflagellates", Academic Press (1984); Henderson, et al., *Phytochemistry* 27:1679-1683 (1988). In contrast, the present invention achieves the cultivation of DHA-producing microorganisms in a simple medium containing glucose and yeast extract. Use of these components in a solution such as seawater provides economically significant growth rates and cell densities. For example, during the course of a 3-5 day fermentation, *C. cohnii* cell densities of at least 10 grams of biomass per liter of solution, and typically from 20 to about 40 grams per liter, can be attained. Such densities have not heretofore been attainable.

Although cultivation can occur in any suitable fermentor, preferably the organism is grown either in a stirred tank fermentor (STF) or in an air lift fermentor (ALF), both types known to those of skill in the art. When a STF is selected, agitation is provided using either Rushton-type high efficiency turbines or pitched-blade or marine impellers. Agitation and duration renews the supply of oxygen to the microorganisms. The rate of agitation normally is increased as the biomass increases, due to the increased demand for oxygen. It is desirable to keep the tip speed at not greater than about 500 cm/sec. Selection of strains of microorganisms which are capable of withstanding greater tip speeds without undergoing shear is within the

purview of those of skill in the art. The use of such strains is expressly included in this invention.

As noted above, seawater is an acceptable medium for the nutrient solution. The seawater can be either natural, filtered or an artificial mix, each of which can be diluted to ¼ strength with tap water or concentrated to 2 times normal strength. A preferred example is Instant Ocean® (IO) brand artificial seawater. Although *C. cohnii* is a marine microorganism, some growth has been observed in zero salinity. The use of variants which grow well in reduced salinities is specifically encompassed by this invention. Micronutrients can be added and may be required. However, such micronutrients are known to those of skill in the art and generally are present in seawater or tap water. If the organism selected is heterotrophic, such as *C. cohnii*, then a carbon source is added.

Preferably, after addition of the seawater medium to the fermentor, the fermentor containing the medium is sterilized and cooled prior to adding the nutrients and a seeding population of microorganism. (Although it is acceptable to sterilize the nutrients together with the seawater, sterilization in this manner can result in a loss of available glucose.) The nutrients and microorganism can be added simultaneously or sequentially.

An effective seed concentration can be determined by those of skill in the art. When a STF is used, the addition of a population of from about .05 to 1.0 grams of dry weight equivalent per liter at the beginning of the fermentation is preferred. This is about 10^5 cells per ml. Thus, for a 30 liter fermentor, 1.5 liters of seeding media, containing viable cells at a density of 20g dry weight per liter would be added.

Oxygen levels preferably are maintained at a D.O. of at least about 10% of air saturation level. Biosynthesis of DHA requires oxygen and, accordingly, higher yields of DHA require D.O. levels at from about 10% to 50% of air saturation levels. Agitation tip speeds of 150-200 cm/sec in combination with an aeration rate of 1 VVM (volume of air/volume of fermentor per minute) provides D.O. levels of from about 20% to about 30% at biomass densities of about 25g dry weight/liter of culture. Higher cell densities may require higher D.O. levels, which can be attained by increased aeration rates by O_2 sparging, or by increasing the air pressure in the fermentor.

Acceptable carbon sources are known to those of skill in the art. For example, carbon can be provided to *C. cohnii* in the form of glucose. Other heterotrophs can use other reduced carbon sources, a matter easily determined by those of skill in the art, and autotrophs utilize carbon dioxide. *C. cohnii* will also grow on other reduced, more complex, carbon sources. Typically, a fermentation is initiated with about 10-20 g/liter glucose. More glucose is added during the fermentation as required. Alternatively, from about 80 to 150g glucose/liter initially can be added, thereby minimizing the frequency of future additions. If glucose levels drop to zero, the culture can die within a few hours. The amount of carbon source provided to other organisms can readily be determined by those of skill in the art.

In addition to a reduced carbon source, a nitrogen source, such as yeast extract (YE), is provided to the medium. Commercially available yeast extract is acceptable. For example, DIFCO brand yeast extract can be used. The yeast extract is an organic nitrogen source also containing micronutrients. Other organic nitrogen sources can easily be determined by those of skill in the art. However, such compounds are more expensive than yeast extract. The use of

variants capable of growing on urea or nitrates is within the scope of this invention. Typically, the fermentation is initiated with about 4-8g YE/liter. More YE can be added as required. A typical fermentation run requires about 25 to 50g YE/liter over the course of the run. Accordingly, that amount of YE can be added initially with a reduced need for further additions. The precise amount can be determined by those of skill in the art.

The cultivation can be carried out at any life-sustaining temperature. Generally *C. cohnii* will grow at temperatures ranging from about 15°C to 34°C. Preferably the temperature is maintained at about 20-28°C. Strains which grow at higher temperatures are preferred, because they will have a faster doubling time, thereby reducing the fermentation time. Appropriate temperature ranges for other microorganisms are readily determined by those of skill in the art.

J7 cont

The cultivation can be carried out over a broad pH range, typically from about pH 5.0 to 9.0. Preferably, a pH range of from about 7.0 to about 7.8 is used. The initial growth tends to acidify the medium. Addition of a base, such as KOH or NaOH, corrects this acidification. During the later stages of the fermentation, the culture medium tends to become alkaline. The addition of YE ordinarily is sufficient to maintain the pH in the desired range. However, if desired, inorganic acid pH controls can be used to correct alkalinity.

Production of the single cell oil is induced in the dinoflagellates by the imposition of a nitrogen deficiency. Such deficiencies are caused by providing YE in a limiting amount such that the medium runs out of YE while available glucose remains. The present invention recognizes that it is the carbon source to nitrogen source ratio which promotes the efficient production of the single cell oil. Using glucose and YE as exemplary, a preferred ratio of

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carbon source to nitrogen source is about 2-4 parts glucose to 1 part YE. Similar ratios for other carbon and nitrogen sources can be calculated by those of skill in the art.

After induction of oil production, the culture is grown for about 24 additional hours. During this period of oleosynthesis, the single cell oil containing DHA is being synthesized and visible oil droplets become apparent. Those of skill in the art can readily calculate the time of fermentation required to achieve the expected amount of cell biomass based upon the added amount of YE. When that time has passed, the culture is grown for an additional 24 hours and harvested. In general the *C. cohnii* are cultivated for a time sufficient to produce single cell oil, usually from about 60 to about 90 hours, although this time is subject to variation.

From about 20 to 30% of the resultant biomass, using wild-type *C. cohnii*, comprises extractable oil. Strain selection can increase this percentage and such selection is within the scope of this invention. Preferably, the oil comprises greater than about 90% triglycerides having, in general, the following fatty acid composition.

15-20% myristic acid ($C_{14:0}$)

20-25% palmitic acid ($C_{16:0}$)

10-15% oleic acid ($C_{18:1}$)

40-45% DHA ($C_{22:6}$)

0-5% others

The crude oil is characterized by a yellow-orange color and is liquid at room temperature. Desirably, the oil contains at least about 20% DHA by weight and most preferably at least about 35% DHA by weight.

The organisms are harvested by conventional means, known to those of skill in the art, such as centrifugation, flocculation or filtration, and can be processed immediately or dried for future processing. In either event, the oil can be extracted readily with an effective amount of solvent. Suitable solvents can be determined by those of skill in the art. However, a preferred solvent is pure hexane. A suitable ratio of hexane to dry biomass is about 4 liters of hexane per kilogram of dry biomass. The hexane preferably is mixed with the biomass in a stirred reaction vessel at a temperature of about 50°C for about 2 hours. After mixing, the biomass is filtered and separated from the hexane containing the oil. The residual biomass, i.e. the single cell edible oil extracted biomass of the microorganisms, such as *C. cohnii*, can be used as an animal feed, containing as it does about 35-40% protein, 8-10% ash and 45-50% carbohydrates. The hexane then is removed from the oil by distillation techniques known to those of skill in the art. Conventional oilseed processing equipment is suitable to perform the filtering, separation and distillation. Additional processing steps, known to those of skill in the art, can be performed if required or desirable for a particular application. These steps also will be similar to those involved in conventional vegetable oil processing and do not comprise a part of this invention.--

Please amend page 7, line 21, by adding the following passage, which corresponds to the text of U.S. Application Serial No. 07/645,454 extending on page 7 from line 1 to line 8 and from page 7, line 11 to page 12, line 5:

--Of those fungal species which previously have had their fatty acids characterized, it has been found that most do not make ARA. Weete, J.D., *Fungal Lipid Biochemistry*, Plenum

Press, N.Y. (1974). Of those species which do make ARA, many, including all previously characterized *Pythium* species, produce significant quantities of eicosapentaenoic acid (EPA) in addition to ARA. Unexpectedly, it has been found that *P. insidiosum* produces ARA without concomitant production of EPA. As with fish oils, high EPA levels in dietary supplements result in a depression of the ability to form ARA from dietary linoleic acid (LOA). Accordingly, while those fungal species producing both ARA and EPA can be utilized in the process of this invention, it is preferable to use species which do not produce significant quantities of EPA. Such preferred species include *Pythium insidiosum* and *Mortierella alpina*. Both species are available commercially and are on deposit with the American Type Culture Collection in Rockville, Maryland, having accession numbers 28251 and 42430, respectively. Throughout this disclosure, unless otherwise expressly stated, *P. insidiosum* will be the representative fungal species.

One of the significant problems which an embodiment of the present invention overcomes, is the depression of ARA biosynthesis in infants caused by the presence of enhanced dietary levels of EPA. This problem can be corrected by providing ARA for use in infant formula at levels substantially similar to those found in human breast milk. Typically in human breast milk, the ratio of ARA:EPA is about 20:1 respectively. The present invention specifically contemplates any microbial oil which provides a sufficient amount of ARA to overcome the negative effects of dietary EPA. Preferably, the use of the ARA-containing oil will result in an ARA:EPA ratio of at least about 5:1. More preferably, the ratio will be at least about 10:1 and, most preferably, it will be at least about 20:1. As can be seen, the higher the amount of ARA in the end product, with respect to the amount of EPA, the more desirable is the result.

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In a process of the present invention, the fungi are cultivated under suitable ARA-containing oil producing cultivating conditions. In general, techniques of fungal cultivation are well known to those of skill in the art and those techniques can be applied to the present inventive process. For example, cultivation of an inoculating amount of fungus can occur in submerged culture in shake flasks. The flask is provided with a growth medium, seeded with fungal mycelium, and grown on a reciprocating shaker for about three to four days.

The composition of the growth medium can vary but always contains carbon and nitrogen sources. A preferred carbon source is glucose, amounts of which can range from about 10-100 grams glucose per liter of growth medium. Typically about 15 grams/liter are utilized for shaker flask culture. The amount can be varied depending upon the desired density of the final culture. Other carbon sources which can be used include molasses, high fructose corn syrup, hydrolyzed starch or any other low cost conventional carbon source used in fermentation processes. Additionally, lactose can be provided as a carbon source for *P. insidiosum*. Thus, whey permeate, which is high in lactose and is a very low cost carbon source, can be used as a substrate. Suitable amounts of these carbon sources can readily be determined by those of skill in the art. Usually, additional carbon needs to be added during the course of the cultivation. This is because the organisms use so much carbon that adding it all in a batch mode could prove unwieldy.

Nitrogen typically is provided in the form of yeast extract at a concentration of from about 2 to about 15 grams extract per liter of growth medium. Preferably, about four grams per liter are provided. Other nitrogen sources can be used, including peptone, tryptone, cornsteep

liquor, etc. The amount to be added of these sources can easily be determined by those of skill in the art. Nitrogen can be added in a batch mode, i.e. all at one time prior to cultivation.

After cultivation for 3-4 days at a suitable temperature, typically about 25-30°C, in amount of fungi has grown which is sufficient for use as an inoculum in a conventional stirred tank fermentor (STF). Such fermentors are known to those of skill in the art and are commercially available. Fermentation can be carried out in batch, fed-batch, or continuous fermentation modes. Preferably, the STF is equipped with a Rushton-type turbine impeller.

The fermentor is prepared by adding the desired carbon and nitrogen sources. For example, a 1.5 liter fermentor can be prepared by mixing about 50 grams of glucose and about 15 grams of yeast extract per liter of tap water. As previously discussed, other carbon or nitrogen sources or mixtures thereof can be used.

The reactor containing the nutrient solution should be sterilized by, for example, heating prior to inoculation. After cooling to about 30°C, the inoculum can be added, and cultivation initiated. Gas exchange is provided by air sparging. The air sparging rate can vary, but preferably is adjusted to from about 0.5 to about 4.0 VVM (volume of air per volume of fermentor per minute). Preferably the dissolved oxygen level is kept at from about 10% to about 50% of the air saturation value of the solution. Accordingly, adjustments in the sparge rate may be required during cultivation. Agitation is desirable. The agitation is provided by the impeller. Agitation tip speed preferably is set within the range of from about 50 cm/sec to about 500 cm/sec, preferably from about 100 to 200 cm/sec.

In general, the amount of inoculum can vary. Typically, from about 2% to about 10% by volume of inoculum can be used. Preferably, in a fermentor seed train about 5% by volume of inoculum can be used.

Nutrient levels should be monitored. When glucose levels drop below 5 g/l, additional glucose should be added. A typical cultivation cycle utilizes about 100 grams of glucose and about 15 grams of yeast extract per liter. It is desirable to deplete the nitrogen during the course of the cultivation as this enhances oil production by the fungi. This is especially true when *M. alpina* is used as the production organism.

Occasionally, the culture will produce an excessive quantity of foam. Optionally, an antifoaming agent, such as those known to those of skill in the art, e.g. Mazu 310®, can be added to prevent foam.

J3 cont

The temperature of cultivation can vary. However, those fungi which produce both ARA and EPA tend to produce less EPA and more ARA when cultivated at higher temperatures. For example, when *Mortierella alpina* is cultivated at less than 18°C, it begins to produce EPA. Thus, it is preferable to maintain the temperature at a level which induces the preferential production of ARA. Suitable temperatures are typically from about 25°C to about 30°C.

Preferably, cultivation continues until a desired biomass density is achieved. A desirable biomass is about 25 g/l of the organism. Such a biomass typically is attained within 48-72 hours after inoculation. At this time, the organisms typically contain about 5-40% complex lipids, i.e. oil, of which about 10-40% is ARA, and can be harvested. .

Harvesting can be done by any suitable method such as, for example, filtration, centrifugation, or spray drying. Because of lower cost, filtration may be preferred.

After harvesting, the mycelial cake can be extracted. The mycelial cake refers to the collection of biomass resulting after harvest. The cake can be loose or pressed, crumbled or uncrumbled. Optionally, the cake can have any residual water removed, as by vacuum drying or lyophilization, prior to extraction. If this option is selected, it is preferable to use nonpolar solvents to extract the ARA-containing oil. While any non-polar extract is suitable, hexane is preferred.

Alternatively, the wet cake (which typically contains about 30-50% solids) can be crumbled and extracted directly using polar solvents such as ethanol or isopropyl alcohol, or supercritical fluid extraction with solvents such as CO_2 or NO. Preferably, the cakes are crumbled prior to extraction. Advantageously, the present invention permits the economical use of supercritical fluid extraction techniques. McHugh, et al., *Supercritical Fluid Extraction*, Butterworth (1986). Such techniques are known to those of skill in the art and include those presently applied, for example, to decaffeinate coffee beans. While the yields from both wet and dry extractions are similar, wet extraction generally is a more economical process.--

IN THE CLAIMS

Please delete claims 87-91 without prejudice to Applicants right to file divisional application(s) containing the claims deleted herein.

Please amend claims 67, 75-77, 80, 82-84 and 92-94 as follows.

~~67.~~ (Twice Amended) A process for supplementing infant formula with DHA and ARA which comprises: